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Signed, this 25<sup>th</sup> day of September 2008,

  
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# FEDERAL REPUBLIC OF GERMANY



## Certificate of Priority for Filing of a Patent Application

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**Title:** Process for the fermentative preparation of L-amino acids  
using strains of the Enterobacteriaceae family

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**The attached papers are a true and accurate reproduction of the original documents for  
this patent application.**

Munich, 21<sup>st</sup> November 2003

**On behalf of the President of the German  
Patent and Trade Mark Office**

*(signature)*

**Process for the fermentative preparation of L-amino acids  
using strains of the Enterobacteriaceae family**

This invention relates to a process for the fermentative preparation of L-amino acids, in particular L-threonine, using strains of the Enterobacteriaceae family in which the open reading frame (ORF) with the designation yjgF is attenuated.

**Prior art**

L-Amino acids, in particular L-threonine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

It is known to prepare L-amino acids by fermentation of strains of Enterobacteriaceae, in particular *Escherichia coli* (*E. coli*) and *Serratia marcescens*. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as e.g. stirring and supply of oxygen, or the composition of the nutrient media, such as e.g. the sugar concentration during the fermentation, or the working up to the product form, by e.g. ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the threonine analogue  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid (AHV), or are auxotrophic for metabolites of regulatory importance and produce L-amino acid, such as e.g. L-threonine, are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of

strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the production. Summarizing information on the cell and molecular biology of *Escherichia coli* and *Salmonella* are to be found in Neidhardt (ed): *Escherichia coli* and *Salmonella*, Cellular and Molecular Biology, 2nd edition, ASM Press, Washington, D.C., USA, (1995).

#### Object of the invention

The object of the invention is to provide new measures for improved fermentative preparation of L-amino acids, in particular L-threonine.

#### Description of the invention

The invention provides a process for the fermentative preparation of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which, in particular, already produce L-amino acids and in which the nucleotide sequence which codes for the *yjgF* ORF or alleles thereof is or are attenuated.

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Threonine is particularly preferred.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity or concentration of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or a gene or allele or ORF which codes for a corresponding enzyme with a low activity

or inactivates the corresponding enzyme or protein or gene or ORF and optionally combining these measures.

Open reading frame (ORF) describes a section of a nucleotide sequence which codes or can code for a protein or polypeptide or ribonucleic acid to which no function can be assigned according to the prior art. After assignment of a function to the nucleotide sequence section in question, it is in general referred to as a gene.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

The process is characterized in that the following steps are carried out:

- a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which the yjgF ORF or nucleotide sequences which code for it is or are attenuated,
- b) concentration of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and
- c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.

The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen

from the genera *Escherichia*, *Erwinia*, *Providencia* and *Serratia*. The genera *Escherichia* and *Serratia* are preferred. Of the genus *Escherichia* in particular the species *Escherichia coli* and of the genus *Serratia* in particular the species *Serratia marcescens* are to be mentioned.

Suitable strains, which produce L-threonine in particular, of the genus *Escherichia*, in particular of the species *Escherichia coli*, are, for example

- *Escherichia coli* H4581 (EP 0 301 572)
- *Escherichia coli* KY10935 (Technical Research Laboratories 61(11): 1877-1882 (1997))
- *Escherichia coli* VNIIGenetika MG442 (US-A-4278,765)
- *Escherichia coli* VNIIGenetika M1 (US-A-4.321.325)
- *Escherichia coli* VNIIGenetika 472T23 (US-A-5,631,157)
- *Escherichia coli* BKIIM B-3996 (US-A-5.175.107)
- *Escherichia coli* kat 13 (WO 98/04715)
- *Escherichia coli* KCCM-10132 (WO 00/09660)

Suitable L-threonine-producing strains of the genus *Serratia*, in particular of the species *Serratia marcescens*, are, for example

- *Serratia marcescens* HNr21 (Applied and Environmental Microbiology 38(6): 1045-1051 (1979))
- *Serratia marcescens* TLr156 (Gene 57(2-3): 151-158 (1987))
- *Serratia marcescens* T-2000 (Applied Biochemistry and Biotechnology 37(3): 255-265 (1992))

Strains from the Enterobacteriaceae family which produce L-threonine preferably have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of: resistance to  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to  $\alpha$ -methylserine, resistance to diaminosuccinic acid, resistance to  $\alpha$ -aminobutyric acid, resistance to borrelidin, resistance to cyclopentane-carboxylic acid, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate, resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a partial and compensatable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine, resistance to threonine raffinose, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid, resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-valine, sensitivity to fluoropyruvate, defective threonine dehydrogenase, optionally an ability for sucrose utilization, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feed back resistant form, enhancement of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feed back resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feed back resistant form, enhancement of phosphoenol pyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.

It has been found that microorganisms of the Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner after attenuation, in particular elimination, of the yjgF gene or open reading frame (ORF).

The nucleotide sequences of the genes or open reading frames (ORF) of Escherichia coli belong to the prior art and can also be found in the genome sequence of Escherichia coli published by Blattner et al. (Science 277: 1453-1462 (1997)).

The yjgF ORF is described, inter alia, by the following data:

Description: open reading frame  
Function: unknown function  
Reference: Wasinger VC. and Humphery-Smith I.; FEMS microbiology letters 169(2): 375-382 (1998)  
Volz K.; Protein science 8(11): 2428-2437 (1999)  
Accession No.: AE000495

The nucleic acid sequences can be found in the databanks of the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (Bethesda, MD, USA), the nucleotide sequence databank of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany or Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima, Japan).

The open reading frames described in the text references mentioned can be used according to the invention. Alleles of the genes or open reading frames which result from the degeneracy of the genetic code or due to "sense mutations" of neutral function can furthermore be used. The use of endogenous genes or open reading frames is preferred.

"Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or open reading frames



alleles or nucleotide sequences present in the population of a species.

To achieve an attenuation, for example, expression of the genes or open reading frames or the catalytic properties of the enzyme proteins can be reduced or eliminated. The two measures can optionally be combined.

The reduction in gene expression can take place by suitable culturing, by genetic modification (mutation) of the signal structures of gene expression or also by the antisense-RNA technique. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The expert can find information in this respect, inter alia, for example, in Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)), in Carrier und Keasling (Biotechnology Progress 15: 58-64 (1999)), Franch and Gerdes (Current Opinion in Microbiology 3: 159-164 (2000)) and in known textbooks of genetics and molecular biology, such as, for example, the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art. Examples which may be mentioned are the works of Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Yano et al. (Proceedings of the National Academy of Sciences of the United States of America 95: 5511-5515 (1998)), Wentz and Schachmann (Journal of Biological Chemistry 266: 20833-20839 (1991)). Summarizing descriptions can be found in known textbooks of genetics and molecular biology, such as e.g. that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Possible mutations are transitions, transversions, insertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, "missense mutations" or "nonsense mutations" are referred to. Insertions or deletions of at least one base pair in a gene lead to "frame shift mutations", which lead to incorrect amino acids being incorporated or translation being interrupted prematurely. If a stop codon is formed in the coding region as a consequence of the mutation, this also leads to a premature termination of the translation. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on generation of such mutations are prior art and can be found in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations in the gene, such as, for example, deletion mutations, can be incorporated into suitable strains by gene or allele replacement.

A common method is the method, described by Hamilton et al. (Journal of Bacteriology 171: 4617-4622 (1989)), of gene replacement with the aid of a conditionally replicating pSC101 derivative pMAK705. Other methods described in the prior art, such as, for example, that of Martinez-Morales et al. (Journal of Bacteriology 181: 7143-7148 (1999)) or that of Boyd et al. (Journal of Bacteriology 182: 842-847 (2000)), can likewise be used.

It is also possible to transfer mutations in the particular genes or mutations which affect expression of the particular genes or open reading frames into various strains by conjugation or transduction.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family to enhance one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate or enzymes of glycolysis or PTS enzymes or enzymes of sulfur metabolism, in addition to the attenuation of the yjgF ORF.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes or open reading frames, using a potent promoter or a gene or open reading frame which codes for a corresponding enzyme or protein with a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

It may be possible at the same time for one or more of the genes chosen from the group consisting of

- the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the pyc gene of Corynebacterium glutamicum which codes for pyruvate carboxylase (WO 99/18228),

- the pps gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231(2): 332-336 (1992)),
- the ppc gene which codes for phosphoenol pyruvate carboxylase (Gene 31: 279-283 (1984)),
- the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- the rhtB gene which imparts homoserine resistance (EP-A-0 994 190),
- the mqo gene which codes for malate:quinone oxidoreductase (WO 02/06459),
- the rhtC gene which imparts threonine resistance (EP-A-1 013 765),
- the thrE gene of Corynebacterium glutamicum which codes for the threonine export protein (WO 01/92545),
- the gdhA gene which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983)),
- the hns gene which codes for the DNA-binding protein HLP-II (Molecular and General Genetics 212: 199-202 (1988)),
- the pgm gene which codes for phosphoglucomutase (Journal of Bacteriology 176: 5847-5851 (1994)),
- the fba gene which codes for fructose biphosphate aldolase (Biochemical Journal 257: 529-534 (1989)),
- the ptsH gene of the ptsHIcrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),

- the ptsI gene of the ptsHIcrr operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- the crr gene of the ptsHIcrr operon which codes for the glucose-specific IIA component of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- the ptsG gene which codes for the glucose-specific IIBC component (Journal of Biological Chemistry 261: 16398-16403 (1986)),
- the lrp gene which codes for the regulator of the leucine regulon (Journal of Biological Chemistry 266: 10768-10774 (1991)),
- the csrA gene which codes for the global regulator Csr (Journal of Bacteriology 175: 4744-4755 (1993)),
- the fadR gene which codes for the regulator of the fad regulon (Nucleic Acids Research 16: 7995-8009 (1988)),
- the iclR gene which codes for the regulator of central intermediate metabolism (Journal of Bacteriology 172: 2642-2649 (1990)),
- the mopB gene which codes for the 10 kd chaperone (Journal of Biological Chemistry 261: 12414-12419 (1986)) and is also known by the name groES,
- the ahpC gene of the ahpCF operon which codes for the small sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92: 7617-7621 (1995)),
- the ahpF gene of the ahpCF operon which codes for the large sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92: 7617-7621 (1995)),

- the *cysK* gene which codes for cysteine synthase A (Journal of Bacteriology 170: 3150-3157 (1988)),
- the *cysB* gene which codes for the regulator of the *cys* regulon (Journal of Biological Chemistry 262: 5999-6005 (1987)),
- the *cysJ* gene of the *cysJIH* operon which codes for the flavoprotein of NADPH sulfite reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
- the *cysI* gene of the *cysJIH* operon which codes for the haemoprotein of NADPH sulfite reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
- the *cysH* gene of the *cysJIH* operon which codes for adenylyl sulfate reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
- the *phoB* gene of the *phoBR* operon which codes for the positive regulator PhoB of the *pho* regulon (Journal of Molecular Biology 190 (1): 37-44 (1986)),
- the *phoR* gene of the *phoBR* operon which codes for the sensor protein of the *pho* regulon (Journal of Molecular Biology 192 (3): 549-556 (1986)),
- the *phoE* gene which codes for protein E of the outer cell membrane (Journal of Molecular Biology 163 (4): 513-532 (1983)),
- the *pykF* gene which codes for fructose-stimulated pyruvate kinase I (Journal of Bacteriology 177 (19): 5719-5722 (1995)),
- the *pfkB* gene which codes for 6-phosphofructokinase II (Gene 28 (3): 337-342 (1984)),

- the *malE* gene which codes for the periplasmic binding protein of maltose transport (Journal of Biological Chemistry 259 (16): 10606-10613 (1984)),
- the *sodA* gene which codes for superoxide dismutase (Journal of Bacteriology 155 (3): 1078-1087 (1983)),
- the *rseA* gene of the *rseABC* operon which codes for a membrane protein with anti- $\sigma$ E activity (Molecular Microbiology 24 (2): 355-371 (1997)),
- the *rseC* gene of the *rseABC* operon which codes for a global regulator of the  $\sigma$ E factor (Molecular Microbiology 24 (2): 355-371 (1997)),
- the *sucA* gene of the *sucABCD* operon which codes for the decarboxylase sub-unit of 2-ketoglutarate dehydrogenase (European Journal of Biochemistry 141 (2): 351-359 (1984)),
- the *sucB* gene of the *sucABCD* operon which codes for the dihydrolipoyltranssuccinase E2 sub-unit of 2-ketoglutarate dehydrogenase (European Journal of Biochemistry 141 (2): 361-374 (1984)),
- the *sucC* gene of the *sucABCD* operon which codes for the  $\beta$ -sub-unit of succinyl-CoA synthetase (Biochemistry 24 (22): 6245-6252 (1985)),
- the *sucD* gene of the *sucABCD* operon which codes for the  $\alpha$ -sub-unit of succinyl-CoA synthetase (Biochemistry 24 (22): 6245-6252 (1985)),
- the *adk* gene which codes for adenylate kinase (Nucleic Acids Research 13(19): 7139-7151 (1985)),
- the *hdeA* gene which codes for a periplasmic protein with a chaperonin-like function (Journal of Bacteriology 175(23): 7747-7748 (1993)),

- the hdeB gene which codes for a periplasmic protein with a chaperonin-like function (Journal of Bacteriology 175(23): 7747-7748 (1993)),
- the icd gene which codes for isocitrate dehydrogenase (Journal of Biological Chemistry 262(22): 10422-10425 (1987)),
- the mglB gene which codes for the periplasmic, galactose-binding transport protein HLP-II (Molecular and General Genetics 229(3): 453-459 (1991)),
- the lpd gene which codes for dihydrolipoamide dehydrogenase (European Journal of Biochemistry 135(3): 519-527 (1983)),
- the aceE gene which codes for the E1 component of the pyruvate dehydrogenase complex (European Journal of Biochemistry 133(1): 155-162 (1983)),
- the aceF gene which codes for the E2 component of the pyruvate dehydrogenase complex (European Journal of Biochemistry 133(3): 481-489 (1983)),
- the pepB gene which codes for aminopeptidase B (Journal of Fermentation and Bioengineering 82: 392-397 (1996)),
- the aldH gene which codes for aldehyde dehydrogenase (E.C. 1.2.1.3) (Gene 99(1): 15-23 (1991)),
- the gene product of the open reading frame (orf) yodA (Accession Number AE000288 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) (Science 277(5331): 1453-1474 (1997))
- the bfr gene which codes for the iron storage homoprotein (bacterioferritin) (Journal of Bacteriology 171(7): 3940-3947 (1989)),



- the *udp* gene which codes for uridine phosphorylase (Nucleic Acids Research 17(16): 6741 (1989)) and
  - the *rseB* gene which codes the regulator of sigmaE factor activity (Molecular Microbiology 24(2): 355-371 (1997)),
- to be enhanced, in particular over-expressed.

The use of endogenous genes or open reading frames is in general preferred. "Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or, respectively, nucleotide sequences present in the population of a species.

It may furthermore be advantageous for the production of L-amino acids, in particular threonine, in addition to the attenuation of the *yjgF* ORF, for one or more of the genes chosen from the group consisting of

- the *tdh* gene which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- the *mdh* gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- the gene product of the open reading frame (orf) *yjfA* (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA), WO 02/29080),
- the gene product of the open reading frame (orf) *ytfP* (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA), WO 02/29080),
- the *pckA* gene which codes for the enzyme phosphoenolpyruvate carboxykinase (WO 02/29080),
- the *poxB* gene which codes for pyruvate oxidase (WO 02/36797)

- the aceA gene which codes for the enzyme isocitrate lyase (Journal of Bacteriology 170: 4528-4536 (1988)),
- the dgsA gene which codes for the DgsA regulator of the phosphotransferase system (Bioscience, Biotechnology and Biochemistry 59: 256-261 (1995)) and is also known under the name of the mlc gene,
- the fruR gene which codes for the fructose repressor (Molecular and General Genetics 266: 332-336 (1991)) and is also known under the name of the cra gene,
- the rpoS gene which codes for the sigma<sup>38</sup> factor (WO 01/05939) and is also known under the name of the katF gene,
- the aspA gene which codes for aspartate ammonium lyase (aspartase) (Nucleic Acids Research 13(6): 2063-2074 (1985)),
- the aceB gene which codes for malate synthase A (Nucleic Acids Research 16(19): 9342 (1988))

to be attenuated, in particular eliminated or for the expression thereof to be reduced.

In addition to attenuation of the yjgF ORF it may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed batch process (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods are described in the textbook by Chmiel

(Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be

employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-amino acids or L-threonine has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry, 30: 1190-1206 (1958)) or it can be carried out by reversed phase HPLC, as described by Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention is used for the fermentative preparation of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

**Patent claims**

1. Process for the preparation of L-amino acids, in particular L-threonine, characterized in that the following steps are carried out:
  - a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which the yjgF ORF or the nucleotide sequence which codes for it are attenuated, in particular eliminated, and
  - b) concentration of the desired L-amino acid in the medium or in the cells of the microorganisms, and
  - c) isolation of the desired L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.
2. Process according to claim 1, characterized in that microorganisms in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.
3. Process according to claim 1, characterized in that microorganisms in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
4. Process according to claim 1, characterized in that the expression of the polynucleotide which codes for the product of the open reading frame yjgF is attenuated, in particular eliminated.
5. Process according to claim 1, characterized in that the regulatory and/or catalytic properties of the polypeptide (enzyme protein) for which the

polynucleotide of the open reading frame yjgF codes are reduced.

6. Process according to claim 1, characterized in that, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which additionally at the same time one or more of the genes chosen from the group consisting of:

- 6.1 the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
- 6.2 the pyc gene which codes for pyruvate carboxylase,
- 6.3 the pps gene which codes for phosphoenol pyruvate synthase,
- 6.4 the ppc gene which codes for phosphoenol pyruvate carboxylase,
- 6.5 the pntA and pntB genes which code for transhydrogenase,
- 6.6 the rhtB gene which imparts homoserine resistance,
- 6.7 the mgo gene which codes for malate:quinone oxidoreductase,
- 6.8 the rhtC gene which imparts threonine resistance,
- 6.9 the thrE gene which codes for the threonine export protein,
- 6.10 the gdhA gene which codes for glutamate dehydrogenase,
- 6.11 the hns gene which codes for the DNA-binding protein HLP-II,

- 6.12 the pgm gene which codes for phosphoglucomutase,
- 6.13 the fba gene which codes for fructose biphosphate aldolase,
- 6.14 the ptsH gene which codes for the phosphohistidine protein hexose phosphotransferase,
- 6.15 the ptsI gene which codes for enzyme I of the phosphotransferase system,
- 6.16 the crr gene which codes for the glucose-specific IIA component,
- 6.17 the ptsG gene which codes for the glucose-specific IIBC component,
- 6.18 the lrp gene which codes for the regulator of the leucine regulon,
- 6.19 the csrA gene which codes for the global regulator Csr,
- 6.20 the fadR gene which codes for the regulator of the fad regulon,
- 6.21 the iclR gene which codes for the regulator of central intermediate metabolism,
- 6.22 the mopB gene which codes for 10 Kd chaperone,
- 6.23 the ahpC gene which codes for the small sub-unit of alkyl hydroperoxide reductase,
- 6.24 the ahpF gene which codes for the large sub-unit of alkyl hydroperoxide reductase,
- 6.25 the cysK gene which codes for cysteine synthase A,
- 6.26 the cysB gene which codes for the regulator of the cys regulon,

- 6.27 the *cysJ* gene which codes for the flavoprotein of NADPH sulfite reductase,
- 6.28 the *cysI* gene which codes for the haemoprotein of NADPH sulfite reductase,
- 6.29 the *cysH* gene which codes for adenylyl sulfate reductase,
- 6.30 the *phoB* gene which codes for the positive regulator PhoB of the *pho* regulon,
- 6.31 the *phoR* gene which codes for the sensor protein of the *pho* regulon,
- 6.32 the *phoE* gene which codes for protein E of the outer cell membrane,
- 6.33 the *pykF* gene which codes for fructose-stimulated pyruvate kinase I,
- 6.34 the *pfkB* gene which codes for 6-phosphofructokinase II,
- 6.35 the *malE* gene which codes for the periplasmic binding protein of maltose transport,
- 6.36 the *sodA* gene which codes for superoxide dismutase,
- 6.37 the *rseA* gene which codes for a membrane protein with anti-sigmaE activity,
- 6.38 the *rseC* gene which codes for a global regulator of the sigmaE factor,
- 6.39 the *sucA* gene which codes for the decarboxylase sub-unit of 2-ketoglutarate dehydrogenase,



- 6.40 the sucB gene which codes for the dihydrolipoyltranssuccinase E2 sub-unit of 2-ketoglutarate dehydrogenase,
- 6.41 the sucC gene which codes for the  $\beta$ -sub-unit of succinyl-CoA synthetase,
- 6.42 the sucD gene which codes for the  $\alpha$ -sub-unit of succinyl-CoA synthetase,
- 6.43 the adk gene which codes for adenylate kinase,
- 6.44 the hdeA gene which codes for a periplasmic protein with a chaperonin-like function,
- 6.45 the hdeB gene which codes for a periplasmic protein with a chaperonin-like function,
- 6.46 the icd gene which codes for isocitrate dehydrogenase,
- 6.47 the mglB gene which codes for the periplasmic, galactose-binding transport protein,
- 6.48 the lpd gene which codes for dihydrolipoamide dehydrogenase,
- 6.49 the aceE gene which codes for the E1 component of the pyruvate dehydrogenase complex,
- 6.50 the aceF gene which codes for the E2 component of the pyruvate dehydrogenase complex,
- 6.51 the pepB gene which codes for aminopeptidase B,
- 6.52 the aldH gene which codes for aldehyde dehydrogenase,
- 6.53 the yodA gene which codes for the gene product of the open reading frame yodA,

6.54 the bfr gene which codes for the iron storage homoprotein,

6.55 the udp gene which codes for uridine phosphorylase, and

6.56 the rseB gene which codes for the regulator of sigmaE factor activity

is or are enhanced, in particular over-expressed, are fermented.

7. Process according to claim 1, characterized in that, for the preparation of L-amino acids, microorganisms of the Enterobacteriaceae family in which additionally at the same time one or more of the genes chosen from the group consisting of:

7.1 the tdh gene which codes for threonine dehydrogenase,

7.2 the mdh gene which codes for malate dehydrogenase,

7.3 the gene product of the open reading frame (orf) yjfA,

7.4 the gene product of the open reading frame (orf) ytfP,

7.5 the pckA gene which codes for phosphoenol pyruvate carboxykinase,

7.6 the poxB gene which codes for pyruvate oxidase,

7.7 the aceA gene which codes for isocitrate lyase,

7.8 the dgsA gene which codes for the DgsA regulator of the phosphotransferase system,

- 7.9 the fruR gene which codes for the fructose repressor,
  - 7.10 the rpoS gene which codes for the sigma<sup>38</sup> factor,
  - 7.11 the aspA gene which codes for aspartate ammonium lyase (aspartase) and
  - 7.12 the aceB gene which codes for malate synthase A
- is or are attenuated, in particular eliminated or reduced in expression, are fermented.

**Abstract**

The invention relates to a process for the preparation of L-amino acids, in particular L-threonine, in which the following steps are carried out:

- a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid and in which the yjgF ORF or the nucleotide sequence which codes for it are attenuated, in particular eliminated,
- b) concentration of the L-amino acid in the medium or in the cells of the bacteria, and
- c) isolation of the L-amino acid.